



DNA SEQUENCES ENCODING PEPTIDE SEQUENCES SPECIFIC FOR THE  
HEPATIC STAGES OF P. FALCIPARUM BEARING EPITOPES CAPABLE OF  
STIMULATING THE T LYMPHOCYTES

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The parasites responsible for malaria in man, including in particular Plasmodium falciparum and Plasmodium vivax to mention only the principal ones among them, exhibit different morphologies in the human host and express different antigens as a function of their localization in the organism of the infected host. The morphological and antigenic differences shown by these parasites during their life cycle in man makes it possible to define at least four distinct stages of development.

The very first stage of development of the parasite in man corresponds to the sporozoite form introduced into the blood of the host by bites of insect carriers of the parasite. The second stage corresponds to the passage of the parasite into the liver and to the infection of the hepatic cells in which the parasites develop to form the hepatic schizonts, which burst to release the hepatic merozoites. The third stage is characterized by the infection of the blood erythrocytes by the asexual forms (merozoites) of the parasite; this erythrocytic form of development of the parasite corresponds to the pathogenic phase of the disease. The fourth stage corresponds to the formation of the sexual forms (or gametocytes) which will become the extracellular gametes in the mosquito.

It is known that very many studies have been undertaken in order to isolate from the strains of parasites infective for a human host polypeptide fractions to provide for the needs of the in vitro diagnosis of malaria by

detection of the corresponding antibodies, on the one hand, and to attempt to vaccinate against malaria, on the other.

For example, libraries of cloned cDNAs derived from the sporozoites of Plasmodium falciparum have been established by ENEA et al. (1984) Science, vol. 225, 628-630. It was recognized that these libraries included clones capable of expressing immunogenic polypeptides containing repetitive units of 4 amino acids specific for the circumsporozoite antigen (of P. falciparum).

However, little work has been done on the hepatic forms of the parasites responsible for malaria. The morphology of the hepatic forms was described for the first time in 1948 on biopsies taken from infected human volunteers (Trans. Roy. Soc. Trop. Med. Hyg., 41, 785 (1948). It was possible to describe an antigen specific for the hepatic stage of P. falciparum in the liver of South American monkeys insensitive to the blood forms of the parasite, but in which the hepatic forms can develop (Am. J Trop. Med. Hyg., 33 (3) 336-341 (1984).

The detection of the localization of the liver-specific antigens (designated hereafter by LS antigens for "Liver Stage" antigens) was carried out by immunofluorescence throughout the maturation steps of the schizont. They are localized at the periphery of the parasite 5 to 40 microns in size; subsequently they are distributed between the cytomers or packets of merozoites, when the schizonts attain between 50 and 100 microns. They are different from the surface antigens of the sporozoites and the antigens shared by the schizonts of the blood and the liver which give an internal immunofluorescence image of the parasite.

Although it is now possible to culture hepatic forms of P. falciparum in human hepatocytes (Science, 227 440 (1985)), the low numbers of mature

forms of the parasite obtained by the in vitro and in vivo culture methods do not allow the biochemical analysis of the antigen produced at the hepatic stage.

It has also been observed that the individuals suffering from malaria possess a very high level of antibodies directed against the LSA. The LS antigens seem to be very potent immunogens, among the most potent of all of the antigens synthesized at the various stages of development of the parasite.

One of the objectives of the present invention is precisely to provide novel compositions for the vaccination of humans against the malaria caused by P. falciparum.

The objective of the invention is also the in vitro diagnosis of the infection of an individual by P. falciparum under more sensitive conditions than present methods allow.

A molecule expressed specifically during the hepatic phase has been identified by screening a library of genomic DNA cloned in an expression vector with polyclonal sera (GUERIN-MARCHAND, C. et al.; Nature, 329, 164-167 (1987)). This molecule represents a part of an antigen called LSA (Liver Stage Specific antigen), and is constituted of repetitive motifs of 17 amino acids and seems to be very immunogenic under the natural conditions of exposure to the disease.

These repetitive motifs of 17 amino acids (SEQ. ID NO.: 1) are represented by the formula :

Leu-Ala-Lys-Glu-Lys-Leu-Gln-X-Gln-Gln-Ser-Asp-Leu-Glu-Gln-Glu-Arg  
in which X is Glu or Gly.

The subject of the invention is peptide sequences specific for the hepatic stages of P. falciparum which bear epitopes capable of stimulating the T lymphocytes (in particular the cytotoxic lymphocytes).

The invention relates more particularly to molecules or to peptide or polypeptide compositions characterized by the presence in their structure of one or more peptide sequences bearing all or part of one or more T epitope(s) (epitopes implicated in the stimulation of the T lymphocytes) and, optionally, other epitopes, in particular B epitopes (epitopes corresponding to the antibodies produced by B lymphocytes), characteristic of the proteins resulting from the infectious activity of *P. falciparum* in the liver cells.

Reference will be made in what follows to the Figures in which:

- Figure 1 presents a recombinant protein (SEQ. ID NO.: 31) of the invention of 316 amino acids, designated hereafter as antigen 536 or protein LSA-R-NR,
- Figure 2 provides the nucleotide sequence (SEQ. ID NO.: 32) of one of the recombinant nucleic acids studied (clone DG536) and which codes for the polypeptide LSA-R-NR,
- Figure 3 presents a polypeptide (SEQ. ID NO.: 24) of the invention of 151 amino acids, designated hereafter as antigen 729S,
- Figure 4 corresponds to the nucleotide sequence (SEQ. ID NO.: 33) of the clone DG729S which codes for the polypeptide of figure 3 (EcoRI linkers in bold type),
- Figure 5 presents the polypeptide sequences of the antigens LSA-TER (SEQ. ID NO.: 23), 729S-NRI (SEQ. ID NO.: 26), 729S- (SEQ. ID NO.: 27), 729S-Rep (SEQ. ID NO.: 28),
- Figure 6 presents the 5' end of the nucleotide sequence of the LSA gene, (SEQ. ID NO.: 34)
- Figure 7 presents the coding sequence of the 5' end of the LSA gene (SEQ. ID NOS.: 35-36 and 37) and the corresponding polypeptide sequence (SEQ. ID NO.: 38),

- Figure 8 describes the 3' end of the LSA gene (SEQ. ID NO.: 39),
- Figure 9 gives the sequence of the 3' end of the LSA gene (SEQ. ID NOS.: 40-41 and 42) as well as the corresponding polypeptide sequence (SEQ. ID NO.: 43),
- Figure 10 repeats the sequences (SEQ. ID NO.: 44-47) given in Figure 9, up to the termination codon stop and the terminal amino acid.

Thus, the present invention relates to any molecule or polypeptide composition bearing at least one peptide sequence bearing all or part of one or more epitopes characteristic of a protein produced in the hepatocytes infected by P. falciparum and more particularly bearing all or part of one or more T epitope(s) of the proteins produced at the hepatic stage of P. falciparum characterized in that this peptide sequence is represented by all or part of the amino acid sequence shown in Figure 9 or Figure 10, and corresponds to the 3' end of the LSA gene.

More particularly, the subject of the invention is any molecule or polypeptide composition bearing at least one peptide sequence bearing all or part of one or more epitopes characteristic of a protein produced in the hepatocytes infected by *P. falciparum* and more particularly bearing all or part of one or more T epitope(s) of the proteins produced at the hepatic stage of *P. falciparum* characterized in that this peptide sequence is represented by all or part of the sequence of the last 279 amino acids shown in Figure 10, this amino acid sequence being optionally preceded by all or part of one or more of the sequences (SEQ. ID NOS.: 2-18) of 17 amino acids of formula:

$X_1DLEQX_2RX_3AKEKLQX_4QQ$   
 $QX_1DLEQX_2RX_3AKEKLQX_4Q$   
 $QQX_1DLEQX_2RX_3AKEKLQX_4$   
 $X_4QQX_1DLEQX_2RX_3AKEKLQ$

QX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEKL  
 LX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEK  
 KLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKE  
 EKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AK  
 KEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>A  
 AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>  
 X<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>R  
 RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>  
 X<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQ  
 QX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLE  
 EQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DL  
 LEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>D  
 DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>

in which

- ° X<sub>1</sub> is "Ser" or "Arg",
- ° X<sub>2</sub> is "Glu" or "Asp"
- ° X<sub>3</sub> is "Arg" or "Leu"
- ° X<sub>4</sub> is "Glu" or "Gly"

Consequently, the invention relates more particularly to any molecule or polypeptide composition bearing at least one peptide sequence bearing all or part of one or more epitopes characteristic of a protein produced in the hepatocytes infected by P. falciparum and more particularly bearing all or part of one or more T epitope(s) of the proteins produced at the hepatic stage of P. falciparum, characterized in that this peptide sequence is represented by all or part of the following amino acid sequence (SEQ. ID NO.: 19)

RKADTKKNLERKKEHGDILAEDLYGRLEIPAIELPS  
 ENERGYYPHQSSLPQDNRGNSRDSKEISIIKTNR

ESITTNVEGRDDIHKGHLEKKDGSIKPEQKEDKS

this amino acid sequence being optionally preceded by all or part of one or more sequences (SEQ. ID NOS.: 2-18) of 17 amino acids of formula:

$X_1DLEQX_2RX_3AKEKLQX_4QQ$   
 $QX_1DLEQX_2RX_3AKEKLQX_4Q$   
 $QQX_1DLEQX_2RX_3AKEKLQX_4$   
 $X_4QQX_1DLEQX_2RX_3AKEKLQ$   
 $QX_4QQX_1DLEQX_2RX_3AKEKL$   
 $LQX_4QQX_1DLEQX_2RX_3AKEK$   
 $KLQX_4QQX_1DLEQX_2RX_3AKE$   
 $EKLQX_4QQX_1DLEQX_2RX_3AK$   
 $KEKLQX_4QQX_1DLEQX_2RX_3A$   
 $AKEKLQX_4QQX_1DLEQX_2RX_3$   
 $X_3AKEKLQX_4QQX_1DLEQX_2R$   
 $RX_3AKEKLQX_4QQX_1DLEQX_2$   
 $X_2RX_3AKEKLQX_4QQX_1DLEQ$   
 $QX_2RX_3AKEKLQX_4QQX_1DLE$   
 $EQX_2RX_3AKEKLQX_4QQX_1DL$   
 $LEQX_2RX_3AKEKLQX_4QQX_1D$   
 $DLEQX_2RX_3AKEKLQX_4QQX_1$

in which:

- °  $X_1$  is "Ser" or "Arg"
- °  $X_2$  is "Glu" or "Asp"
- °  $X_3$  is "Arg" or "Leu"
- °  $X_4$  is "Glu" or "Gly"

Thus the present invention relates in particular to the peptide sequence shown in Figure 1. This sequence is constituted of 316 amino

acids. At the 5' end 209 amino acids are found organized in repeats of 17 amino acids corresponding to the formulae indicated above. At the 3' end, repeats totalling 107 amino acids are found.

The invention relates more particularly to any polypeptide characterized by all or part of the following amino acid sequence (SEQ. ID NO.: 20):

LQEQQRDLEQRKADTKKNLERKKEHGDILAEDLYGRLEIPAIELPSENERGY  
IPHQSSLPQDNRGNSRDSKEISIIKTNRESITTNVEGRRDIHKHLEEKDGD  
SIKPEQKEDKS

A preferred polypeptide of the invention is represented by all or part of the following amino acid sequence (SEQ. ID NO.: 21):

DTKKNLERKKEHGDILAEDLYGRLEIP

(this polypeptide being designated hereafter by the expression LSA-NR (LSA-non-repeated), or also by any sequence derived from the preceding sequence and modified by the substitution of maximally 40% of the amino acids while retaining its physiological activity such as the induction of a response of the T lymphocytes, in particular the cytotoxic T lymphocytes.

Another particularly preferred polypeptide of the invention is characterized by all or part of the following amino acid sequence (SEQ. ID NO.: 22):

ERRAKEKLQEQQRDLEQRKADTKK

(this polypeptide being designated hereafter by the expression LSA-J, or LSA-junction, since it overlaps the repetitive part and the non-repetitive part of the molecule shown in Figure 1).

Another preferred peptide (SEQ. ID NO.: 23), designated LSA-TER, is the following:

NSRDSKEISIIKTNRESITTNVEGRRDIHK



These last three polypeptides are more particularly useful on account of the amphipaticity which characterizes them, and because of their three dimensional conformation according to the predictions made by the procedure of Chou and Fassmann.

The subject of the invention is also any molecule or polypeptide composition bearing at least one peptide sequence bearing all or part of one or more epitope(s) characteristic of a protein produced at the sporozoite, hepatic and blood (erythrocytic) stages of P. falciparum and more particularly bearing one or more T epitopes, characterized in that this peptide sequence is represented by all or part of the following amino acid sequence (SEQ. ID NO.: 24):

RDELFNELLNSVDVNGEVKENILEESQVNDDIFNSLVKSVQQEQQHNVEEKVE  
ESVEENDEESVEENVEENVEENDDGGSVASSVEESIASSVDESIDSIEENVAP  
TVEEIIIVAPTVEEIVAPSVVEKCAPSVEESVAPSVEESVAEMLKER

shown in Figure 3 and designated hereafter as the polypeptide 729S.

More particularly, the subject of the invention is the amino acid sequence derived from the preceding sequence and characterized by all or part of the following amino acid sequence:

RDELFNELLNSVDVNGEVKENILEESQVNDDIFNSLVKSVQQEQQHN  
(SEQ. ID NO.: 25)

According to another advantageous embodiment of the invention, sequences (SEQ. ID NOS.: 26-28) of interest derived from the amino acid sequence of the polypeptide 729S are the following:

- DELFNELLNSVDVNGEVKENILEESQ,
- LEESQVNDDIFNSLVKSVQQEQQHN,
- VEKCAPSVEESVAPSVEESVAEMLKER.

These sequences are designated 729S-NRI, 729S-NRII, 729S-Rep, respectively.

The subject of the invention is also any molecule or polypeptide composition comprising at least one peptide sequence bearing all or part of one or more epitopes characteristic of a protein produced in the hepatocytes infected by P. falciparum, characterized in that this peptide sequence is represented by all or part of the amino acid sequence shown in Figure 7, and corresponds to the 5' end of the LSA gene.

Consequently, the subject of the invention is more particularly any molecule or polypeptide composition comprising a least one peptide sequence bearing all or part of one or more epitopes characteristic of a protein produced in the hepatocytes infected by P. falciparum and bearing more particularly all or part of one or more T epitope(s) of the proteins produced at the hepatic stage of P. falciparum, characterized in that this peptide sequence is represented by all or part of the sequence of the first 153 amino acids shown in Figure 7, this amino acid sequence being optionally followed by all or of one or more sequences (SEQ. ID NOS.: 2-18) of 17 amino acids of formula:

$X_1DLEQX_2RX_3AKEKLQX_4QQ$   
 $QX_1DLEQX_2RX_3AKEKLQX_4Q$   
 $QQX_1DLEQX_2RX_3AKEKLQX_4$   
 $X_4QQX_1DLEQX_2RX_3AKEKLQ$   
 $QX_4QQX_1DLEQX_2RX_3AKEKL$   
 $LQX_4QQX_1DLEQX_2RX_3AKEK$   
 $KLQX_4QQX_1DLEQX_2RX_3AKE$   
 $EKLQX_4QQX_1DLEQX_2RX_3AK$   
 $KEKLQX_4QQX_1DLEQX_2RX_3A$   
 $AKEKLQX_4QQX_1DLEQX_2RX_3$   
 $X_3AKEKLQX_4QQX_1DLEQX_2R$

RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>  
 X<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQ  
 QX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLE  
 EQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DL  
 LEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>D  
 DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>

in which

- ° X<sub>1</sub> is “Ser” or “Arg”
- ° X<sub>2</sub> is “Glu” or “Asp”
- ° X<sub>3</sub> is “Arg” or “Leu”
- ° X<sub>4</sub> is “Glu” or “Gly”

The invention also relates to any molecule or polypeptide composition comprising at least one peptide sequence bearing all or part of one or more epitopes characteristic of a protein produced in the hepatocytes infected by P. falciparum and bearing more particularly all or part of one or more T epitope(s) of the proteins produced at the hepatic stage of P. falciparum, characterized in that this peptide sequence comprises successively:

- all or part of the sequence of the first 153 amino acids shown in figure 7,
- optionally, all or part of one or more of the sequences (SEQ. ID NOS.: 2-18) of 17 amino acids of formula:

X<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQ  
 QX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>Q  
 QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>  
 X<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQ  
 QX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEKL  
 LX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKEK

KLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AKE  
 EKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>AK  
 KEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>A  
 AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>RX<sub>3</sub>  
 X<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>R  
 RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQX<sub>2</sub>  
 X<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLEQ  
 QX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DLE  
 EQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>DL  
 LEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>D  
 DLEQX<sub>2</sub>RX<sub>3</sub>AKEKLQX<sub>4</sub>QQX<sub>1</sub>

in which

- ° X<sub>1</sub> is “Ser” or “Arg”
- ° X<sub>2</sub> is “Glu” or “Asp”
- ° X<sub>3</sub> is “Arg” or “Leu”
- ° X<sub>4</sub> is “Glu” or “Gly”

- and all or part of the last 279 amino acids shown in Figure 10.

The invention also relates to any polypeptide composition constituted by several different peptide sequences which bear all or part of one or more epitope(s) characteristic of a protein produced in the hepatocytes infected by P. falciparum, as described above.

Generally speaking, by all or part of a peptide sequence of the invention is meant any sequence comprising a least 4 to 5 amino acids up to the maximal number of amino acids of the sequences described above.

It will be obvious that the free reactive functions that some amino acids included in the composition of the molecules according to the invention may possess, in particular the free carboxyl groups borne by the

Glu residues or by the C-terminal amino acid, on the one hand, and/or the free groups borne by the N-terminal amino acid or by amino acids within the peptide chain, for example Lys, on the other, may be modified, provided that this modification does not lead to a modification of the antigenic properties, or, according to circumstances, immunogenic properties of the whole molecule. The molecules thus modified are automatically included in the framework of protection given to the invention by the Claims. These carboxyl functions may possibly be acylated or esterified.

Other modifications are also included in the framework of the invention. In particular, the amine or ester functions of the terminal amino acids, or both at once, may themselves be linked to other amino acids. For example, the N-terminal amino acid may be linked to a sequence comprising from one to several amino acids corresponding to a part of the C-terminal region of another peptide conforming to the definition which was given to it above, or vice versa.

It will also be obvious that any peptide sequence derived from the modification by substitution and/or by addition and/or deletion of one or more amine acids of one of the peptide sequences described above is included in the framework of protection given to the invention by the Claims, provided that this modification does not impair the antigenic or immunogenic properties of the polypeptides of the invention, in particular when these immunogenic properties have been reinforced adequately, for example by combination of these polypeptides with a suitable immunological adjuvant (for example, a muramyl peptide) or by coupling to a carrier molecule of higher molecular weight (for example, a serum albumin or a polylysine) or a toxin of the tetanus type or another antigen of P. falciparum

More particularly, the invention relates to any peptide sequence derived from the peptide sequences mentioned above, and exhibiting modifications resulting from substitution of maxima 40% of the amino acids while retaining the biological activity of the sequences of the invention, namely in particular the induction of a response of the T lymphocytes, in particular the cytotoxic T lymphocytes.

The invention relates more generally to any molecule characterized by the presence in its structure of one or more peptide sequences which exhibit immunological cross-reactions with the peptide sequences corresponding to the preceding formulae towards antibodies which can be induced by these latter in vivo.

The invention also relates to any sequence of nucleotides which codes for a polypeptide of the invention and, more particularly, any sequence of nucleotides corresponding to one of the amino acid sequences of the invention according to the universal genetic code.

The subject of the invention is more particularly the nucleotide sequence constituted by the 951 nucleotides shown in Figure 2, and which code for the above-mentioned polypeptide of 316 amino acids (also designated hereafter by the recombinant protein LSA-R-NR) shown in Figure 1.

The invention also relates to the nucleotide sequence shown in Figure 8, which corresponds to the 3' end of the LSA gene.

The invention also relates to the nucleotide sequences which code for peptide subsequences of the invention. Particular mention should be made of the nucleotide sequences delimited by the nucleotides situated at the positions 630 to 949, 597 to 648 (coding for the peptide LSA-J) or 640 to 717 (coding for the peptide LSA-NR) of Figure 2.

The invention also relates to all or part of the nucleotide sequence of Figure 4 which codes for all or part of the peptide sequence 729S shown in Figure 3.

The subject of the invention is also the nucleotide sequence shown in Figure 5, which corresponds to the 5' end of the LSA gene.

The invention also relates to any sequence of nucleotides which codes for a polypeptide identical with, or one similar from the point of view of both structure and antigenic properties to, those of the invention, this sequence being capable of hybridizing with all or part of the nucleotide sequence defined by the nucleotides situated at the positions 597 to 949 of figure 2, or with all or part of the nucleotide sequence of Figure 4 or the sequences complementary to these latter, under the following conditions:

- pre-treatment (pre-hybridization) of the nitrocellulose filter supporting the nucleic acid fragment to be tested with hybridization buffer (composed of 6 SSC, 5x Denhardt's, 0.5% SDS, 100 ug/l denatured, sonicated salmon sperm DNA) this operation being carried out at 65°C for 1 hour;

- replacement of the hybridization buffer in contact with the support to which the nucleic acid fragment is now bound by hybridization buffer of the same composition and addition of the above-mentioned sequence shown in Figure 2 or Figure 4 as probe, in particular radioactively labelled, and denatured beforehand;

- incubation of the said nucleic acid fragment bound to the support in this incubation buffer with the above-mentioned sequence shown in Figure 2 or Figure 4 at 65°C for a period of about 1 hour;

- the removal of the buffer containing the probe not bound by two successive washings of 30 minutes each with a buffer solution composed of 2 x SSC and 0.5% SDS at 65°C.

It should be recalled that 20 x SSC = 175.3 g NaCl, 88.2 g trisodium citrate/l, pH 7; Denhardt's 50 x = 5 g Ficoll 400 , 5 g polyvinyl pyrrolidone, 5 g BSA (bovine serum albumin) fraction V/l; SDS is sodium dodecyl sulfate.

The subject of the invention is any recombinant nucleic acid containing at least one nucleotide sequence of the invention inserted into a nucleic acid heterologous with respect to the said nucleotide sequence.

The invention relates more particularly to a recombinant nucleic acid such as that defined above in which the nucleotide sequence of the invention is preceded by a promoter (in particular, an inducible promoter) under the control of which transcription of the said sequence is capable of being carried out and optionally followed by a sequence coding for signals for the termination of transcription.

The invention relates to any recombinant vector, used in particular for cloning a nucleotide sequence of the invention and/or the expression of the polypeptide encoded in this sequence, and characterized in that it contains a recombinant nucleic acid such as that defined above at one of its sites inessential for its replication.

As an example of a vector mentioned above, mention should be made of plasmids, cosmids or phages.

Consequently, the invention relates more particularly to the plasmid DG3536 deposited with the CNCM under the No. I-1027 on 17 January 1991, as well as the plasmid DG729S deposited with the CNCM under the No. I-1028 on 17 January 1991.



The subject of the invention is also a procedure for the preparation of a polypeptide of the invention by transformation of a cell host with the aid of a recombinant vector of the type indicated above, followed by the placing of the thus transformed cell host in culture and the recovery of the polypeptide from the culture medium.

Thus, the invention relates to any cell host transformed by a recombinant vector as defined above and which comprises the regulatory elements which allow the expression of the nucleotide sequence coding for a polypeptide according to the invention.

The subject of the invention is more particularly DNA (or RNA) primers which can be used in the context of the synthesis of nucleotide and/or polypeptide sequences according to the invention by the PCR (Polymerase Chain Reaction) procedure, as described in the American patents No. 4,683,202 and No. 4,683,195 and the European patent application No. 200.362 (PCR = assembly-line amplification of the DNA).

The invention relates to any DNA or RNA primer, characterized in that it is constituted of about 10 to 25 nucleotides identical with the first 10 to 25 nucleotides of the nucleotide sequence which codes for a peptide sequence according to the invention or identical with the last 10 to 25 nucleotides of the said sequence.

The invention also relates to any DNA or RNA primer, characterized in that it is constituted of about 10 to 25 nucleotides complementary to the first 10 to 25 nucleotides of the nucleotide sequence according to the invention or complementary to the last 10 to 25 nucleotides of the said nucleotide sequence.

The subject of the invention is also any DNA or RNA primer, characterized in that it is constituted of about 10 to 25 nucleotides which are

capable of hybridizing with the first 10 to 25 nucleotides or with the last 10 to 25 nucleotides of the said nucleotide sequence which codes for a polypeptide of the invention under the conditions of hybridization defined above.

Thus the present invention relates more particularly to a procedure for the preparation of a polypeptide of the invention comprising the following steps:

- optionally, the prior amplification according to the PCR procedure of a quantity of the nucleotide sequence which codes for the said polypeptide with the aid of two DNA primers selected such that one of these primers is identical with the first 10 to 25 nucleotides of the nucleotide sequence which codes for the said polypeptide, whereas the other primer is complementary to the last 10 to 25 nucleotides (or hybridizes with these last 10 to 25 nucleotides) of the said nucleotide sequence, or conversely such that one of these primers is identical with the last 10 to 25 nucleotides of the said sequence, whereas the other primer is complementary to the first 10 to 25 nucleotides (or hybridizes with these first 10 to 25 nucleotides) of the said nucleotide sequence, followed by the introduction of the said nucleotide sequences thus amplified into a suitable vector,

- the placing in culture in a suitable culture medium of a cell host previously transformed by a suitable vector containing a nucleic acid according to the invention containing the nucleotide sequence which codes for the said polypeptide, and

- the recovery of the polypeptide produced by the said transformed cell host from the above-mentioned culture medium.

As examples of DNA or RNA primers according to the invention, mention should be made of the following sequences (SEQ. ID NOS.: 29-30):

3' ->5 TTTCGCTAGATCTTGTT & TCTAAATAGAAGAAA

The peptides according to the invention may also be prepared by the standard procedures used in the field of peptide synthesis. Such synthesis may be carried out in homogeneous solution or on a solid phase.

For example, recourse may be had to the synthetic procedure in homogeneous solution described by HOUBEMWEYL in the monograph entitled "Methoden der Organischen Chemie" (Methods of Organic Chemistry) edited by E. Wunsch, vol. 15-I and II, THIEME, Stuttgart 1974.

This method of synthesis consists of successively condensing the successive aminoacyl residues in the required order, or of condensing aminoacyl residues and fragments previously formed which already contain several amino acids in the correct order, or also of condensing several fragments thus prepared beforehand, it being understood that care will be taken to protect beforehand all of the reactive functions borne by these aminoacyl residues or fragments, with the exception of the amino function of the one and the carboxyl function of the other or vice versa, which are usually required to participate in the formation of the peptide bonds, in particular after activation of the carboxyl function according to the methods well known in peptide synthesis. As a variant, it will also be possible to have recourse to coupling reactions which make use of standard coupling reagents of the carbodiimide type, such as for example 1-ethyl-3- (3 - dimethylaminopropyl)-carbodiimide.

When the amino acyl residue used possesses an additional acidic function (in particular in the case of glutamic acid), such functions should be protected for example by t-butyl ester groups.

In the case of stepwise synthesis, the amino acids being added one at a time, the synthesis begins preferably with the condensation of the C-

terminal amino acid with the amino acid which corresponds to the neighbouring aminoacyl residue in the desired sequence and so on, one after the other, until the N-terminal amino acid is reached.

According to another preferred procedure of the invention, recourse is had to that described by K. B. MERRIFIELD in the article entitled "Solid phase peptide synthesis" (J. Am. Chem. Soc., 45, 2149-2154).

In order to synthesize a peptide chain according to the MERRIFIELD procedure, recourse is had to a very porous polymeric resin to which the first, C-terminal amine acid of the chain is attached. This amino acid is attached to the resin through the intermediary of its carboxyl group and its amino function is protected, for example by means of the t-butoxycarbonyl group.

When the first, C-terminal amino acid has thus been attached to the resin, the protecting group of the amino function is removed by washing the resin with acid.

In the case in which the protecting group of the amine function is the t-butoxycarbonyl group, it may be removed by treatment of the resin with trifluoroacetic acid.

The second amino acid is then coupled to the deprotected amine function of the first C-terminal amino acid to furnish the second aminoacyl residue of the desired sequence, counting from the C-terminus. Preferably, the carboxyl function of this second amino acid is activated for example by means of dicyclohexylcarbodiimide and the amine function is protected, for example by means of t-butoxycarbonyl.

The first part of the desired peptide chain is thus produced, which contains two amino acids and the terminal amino function of which is protected. As previously, the amine function is deprotected and it is then

possible to proceed to the attachment of the third aminoacyl residue under conditions analogous to those for the addition of the second, penultimate C-terminal amino acid.

In this way, the amino acids which will constitute the peptide chain are added one after the other to the previously deprotected amine group of the portion of the peptide chain already formed which is attached to the resin.

When the desired peptide chain has been assembled in its entirety, the protecting groups of the different side chains of the amino acids constituting the peptide chain are removed and the peptide is cleaved from the resin, for example with the aid of hydrogen fluoride.

The invention also relates to water-soluble oligomers of the monomeric peptides indicated above.

The oligomerization may cause an increase in the immunogenicity of the monomeric peptides according to the invention. Without such numerical values being considered as limiting, it should nonetheless be mentioned that these oligomers may contain, for example, from 2 to 10 monomeric units.

In order to carry out the oligomerization, recourse may be had to any polymerization procedure commonly used in the field of peptides, this polymerization being conducted until an oligomer or polymer is obtained which contains the required number of monomeric motifs for the acquisition of the desired immunogenicity.

One method of oligomerization or polymerization of the monomer consists in the reaction of the latter with a cross-linking agent such as glutaraldehyde.

It is also possible to have recourse to other methods of oligomerization or coupling, for example to that making use of the

successive coupling of monomeric units through the intermediary of their terminal carboxyl and amino functions in the presence of homo- or hetero bifunctional coupling agents.

The invention also relates to the conjugates obtained by covalent coupling of the peptides according to the invention (or the above mentioned oligomers) to carrier molecules (natural or synthetic), physiologically acceptable and non-toxic, through the intermediary of complementary reactive groups borne respectively by the carrier molecule and the peptide. Examples of suitable groups are illustrated in what follows:

As examples of carrier molecules or macromolecular supports forming part of the composition of the conjugates according to the invention, mention should be made of naturally occurring proteins such as tetanus toxoid, ovalbumin, serum albumin, hemocyanins, the PPD of tuberculin (PPD : "Purified Protein Derivative"), etc...

Mention should be made, for example, of polylysines or poly (D-L-alanine) -poly (L-lysine) as examples of synthetic macromolecular supports.

The literature mentions other types of macromolecular supports which can be used and which usually have a molecular weight higher than 20,000.

In order to synthesize the conjugates according to the invention, recourse may be had to known procedures such as that described by FRANTZ and ROBERTSON in *Infect. and Immunity*, 33, 193-198 (1981) or that described in *Applied and Environmental Microbiology*, (October 1981), vol. 42, No. 4, 611-614 by P. E. KAUFFMAN by using the peptide and the appropriate carrier molecule.

In practice, the following compounds, cited in a non-limiting manner, are advantageously used as coupling agents : glutaraldehyde, ethyl chloroformate, water-soluble carbodiimides : N-ethyl-N' (3-dimethylamino-

propyl) carbodiimide HCl, diisocyanates, bis-diazobenzidine, di- and tri-chloro-s-triazines, cyanogen bromide as well as the coupling agents mentioned in Scand. J. Immunol., (1978), vol. 8, p. 7-23 (AVRAMEAS, TERNYNCK, GUESDON).

It is possible to have recourse to any coupling procedure implicating, on the one hand, one or more reactive functions of the peptide and, on the other, one or more reactive functions of the molecular supports.

Advantageously, these are carboxyl and amine functions which can give rise to a coupling reaction in the presence of a coupling agent of the type used in the synthesis of proteins, for example, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, N-hydroxybenzotriazole, etc.... It is possible to have recourse to glutaraldehyde, in particular when it is required to link together amino groups borne by the peptide and the molecular support, respectively.

The nucleic acids of the invention can be prepared either by a chemical procedure or by other procedures.

A suitable method of preparation of the nucleic acids containing a - maximum of 200 nucleotides (or 200 bp when double-stranded nucleic acids are concerned) of the invention comprises the following steps:

- the synthesis of DNA using the automated beta-cyanoethylphosphoramidite method described in Bio-organic Chemistry 4; 274-325 (1986),
- the cloning of the nucleic acids thus obtained in a suitable vector and the recovery of the nucleic acid with a suitable probe.

A method of preparation by the chemical route of nucleic acids longer than 200 nucleotides (or 200 bp when double-stranded nucleic acids are concerned) of the invention comprises the following steps.

- the assembly of chemically synthesized oligonucleotides, provided at their ends with different restriction sites, the sequences of which are compatible with the amino acid sequence of the natural polypeptide according to the principle described in Proc. Natl. Acad. Sci. USA, 80; 7461-7465, (1983),

- the cloning of the nucleic acids thus obtained in a suitable vector and the recovery of the desired nucleic acid by means of hybridization with a suitable probe.

The nucleic acids of the invention can be prepared in the following manner

- incubation of the genomic DNA isolated from a strain of P. falciparum with DNase I, then addition of EDTA and purification by extraction with the mixture phenol/chloroform/isoamyl alcohol (25/24/1), then with ether,

- treatment of the DNA thus extracted with the EcoRI methylase in the presence of DTT, and purification by extraction as described above,

- incubation of the DNA thus purified with the 4 deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP in the presence of T4 DNA polymerase and DNA ligase of E. coli, followed by purification according to the method described above,

- the cloning of the nucleic acids thus obtained in a suitable vector and the recovery of the desired nucleic acid with the aid of a suitable probe.

The nucleotide probes used for the recovery of the desired nucleic acid in the procedures mentioned above are usually composed of 40 to 200 nucleotides of the nucleotide sequence shown in Figure 2 (selected more particularly from those situated between the positions 597 to 949 shown in Figure 2) or in Figure 4 or its complementary sequence, and are capable of



hybridizing with the desired nucleic acid under the conditions of hybridization defined above. The synthesis of these probes is carried out according to the automated beta-cyanoethylphosphoramidite method described in Bio-organic Chemistry 4, 274-325 (1986).

The molecules according to the invention possess antigenic properties characteristic of the antigens which bear T epitopes, and optionally B epitopes, and which are either specific for the hepatic stage of the development of *P. falciparum* or specific for the sporozoite, hepatic and blood stages, simultaneously.

In fact, as will be described more particularly with the aid of examples of molecules according to the invention in the detailed description which follows, the molecules according to the invention which contain all or part of the amino acid sequence comprised between the positions 200 and 316 shown in Figure 1, react specifically with the antibodies or the lymphocytes directed against the B and/or T epitopes of the antigens produced at the hepatic stage of *P. falciparum* but not with the antibodies directed against other antigens produced by *P. falciparum* or against antigens produced by other species of *Plasmodium*.

These molecules according to the invention thus recognize specifically the antibodies produced by the immune system of an individual infected by *P. falciparum* under the influence of the LSA antigen, the strongly immunogenic character of which has already been mentioned.

The molecules according to the invention comprising all or part of the peptide sequence shown in Figure 3 are not recognized by the former antibodies which react specifically with all or part of the polypeptide defined by the amino acids situated at the positions 200 to 316 in Figure 1.

On the other hand, the polypeptides corresponding to all or part of the peptide sequence shown in figure 3 are recognized by antibodies which react specifically with antigens localized on the surface of sporozoites (derived from different strains of P. falciparum as well as with antigens of the hepatic schizonts and the blood schizonts, and finally with the surface of the sporozoites of P. yoelii but not of P. berghei.

It should also be emphasized that the antibodies which recognize specifically the polypeptides corresponding to all or part of the peptide sequence shown in Figure 3 are capable of blocking completely the entry of the sporozoites of P. yoelii into hepatic cells of rodents in vitro unlike the antibodies directed against the circumsporozoite protein of P. yoelii and of P. falciparum.

The possibility of producing molecules according to the invention in large amounts as well as their properties of specific recognition of antibodies included among the most actively produced on infection of an individual by P. falciparum make the said molecules the reagents of choice for the in vitro diagnosis of malaria in an individual infected by P. falciparum.

The invention thus relates to a procedure for the in vitro detection of antibodies which correlate with malaria originating from the infection of an individual by P. falciparum in a tissue or biological fluid likely to contain them, this procedure comprising the placing of this tissue or biological fluid in contact with a molecule according to the invention under conditions which allow an in vitro immunological reaction between the said molecules and the antibodies possibly present in the tissue or biological fluid to occur, and the in vitro detection of the antigen-antibody complexes possibly formed.

The biological fluid is preferably constituted by a human serum.

Any standard procedure may be used to carry out such a detection.

As an example, a preferred method makes use of immunoenzymatic processes according to the ELISA procedure, or immunofluorescent or radioimmunological (RIA) or equivalent procedures.

Thus, the invention also relates to any molecule according to the invention labelled with the aid of a suitable label of the enzymatic, fluorescent, radioactive, etc... type.

Such methods comprise for example the following steps:

- the loading of defined quantities of a polypeptide composition according to the invention into the wells of a microtitration plate,
- introduction into the said wells of increasing dilutions of serum requiring diagnosis,
- incubation of the microplate,
- repeated rinsings of the microplate,
- introduction into the wells of the microplate labelled antibodies against the immunoglobulins of the blood, the labelling of these antibodies having been carried out with the aid of an enzyme selected from those which are capable of hydrolysing a substrate and of thus changing the absorption of the latter, at least at one particular wavelength,
- detection of the quantity of substrate hydrolysed, in comparison with a control.

The invention also relates to kits for the in vitro diagnosis of malaria caused by P. falciparum which contain:

- a polypeptide composition according to the invention,
- the reagents for the constitution of the medium suitable for carrying out the immunological reaction,
- the reagents necessary for the detection of the antigen- antibody complexes produced by the immunological reaction, these reagents may also

be labelled, or be capable of being recognized in turn by a labelled reagent, more particularly in the case in which the above-mentioned polypeptide composition is not labelled.

- a reference tissue or biological fluid lacking antibodies recognized by the above-mentioned polypeptide composition.

The invention relates to the antibodies themselves formed again the polypeptides of the invention.

It will be obvious that these antibodies are not limited to polyclonal antibodies.

The invention also applies to any monoclonal antibody produced by any hybridoma capable of being formed by standard methods from the spleen cells of an animal, in particular a mouse or a rat, immunised against one of the purified peptides of the invention, on the one hand, and cells of a suitable myeloma cell line, on the other, and which can be selected for its capacity to produce monoclonal antibodies which recognize the polypeptide initially used for the immunization of the animals.

The invention also relates to a method for the in vitro diagnosis of malaria in an individual likely to be infected by P. falciparum which comprises the placing of a tissue or biological fluid taken from an individual in contact with antibodies such as those described above under conditions which allow an in vitro immunological reaction between the said antibodies and the proteins specific for P. falciparum possibly present in the biological tissue to occur, and the in vitro detection of the antigen antibody complexes possibly formed.

Consequently, the subject of the invention is a kit for the in vitro diagnosis of malaria containing:

- antibodies such as those described above,

- the reagents for making up to appropriate medium for carrying out the immunological reaction,

- the reagents making possible the detection of the antigen-antibody complexes produced by the immunological reaction, these reagents may also be labelled, or be capable of being recognized in turn by a labelled reagent, more particularly in the case in which the above-mentioned polypeptide composition is not labelled.

The invention also relates to a nucleotide detection probe characterized in that it is composed of all or part of one of the nucleotide sequences of the invention such as defined above.

More particularly the subject of the invention is an in vitro diagnostic method for malaria in an individual likely to be infected by P. falciparum which comprises the following steps:

- possibly prior amplification of the quantity of nucleotide sequences according to the invention likely to be contained in the biological sample taken from the said individual, with the aid of two DNA primers selected in the manner indicated above,

- the placing of the above-mentioned biological sample in contact with a nucleotide probe such as that defined above under conditions which allow the production of a hybridization complex formed between the said probe and the said nucleotide sequence,

- the detection of the above-mentioned hybridization complex possibly formed.

As examples of nucleotide probes of the invention, mention should be made of the following sequences (SEQ. ID NOS.: 29-30):

3' ->5' : TTTCGCTAGATCTTGTT & TCTAAATAGAAGAAA

Most especially, the invention opens the door to the development of novel vaccinating principles against malaria originating from the infection of an individual by P. falciparum.

The invention also relates to the compositions prepared in the form of vaccines which contain either one or more peptides according to the invention or an oligomer of this or these peptide(s) or also a conjugate of this or these peptide(s) or oligomer with a carrier molecule, in combination with a suitable pharmaceutically acceptable vehicle and, optionally, with other active ingredients which vaccinate against malaria.

A particularly useful pharmaceutical composition of the invention is characterized in that it contains all or part of the peptide sequence defined by the amino acids situated at the positions 200 to 316 of Figure 1, in combination with all or part of the peptide sequence shown in Figure 3.

Advantageous pharmaceutical compositions are constituted by solutions, suspensions or injectable liposomes containing an efficacious dose of at least one product according to the invention. Preferably, these solutions, suspensions or liposomes are prepared in an isotonic sterilized aqueous phase, preferably a saline or glucose solution.

The invention relates more particularly to such suspensions, solutions or liposomes which can be administered by intradermal, intramuscular or subcutaneous injection or even by scarification.

It also relates to pharmaceutical compositions which can be administered by other routes, in particular the oral or rectal route, or also in the form of aerosols designed to come into contact with mucous membranes, in particular the ocular, nasal, pulmonary or vaginal mucous membranes.

Consequently, it relates to pharmaceutical compositions in which at least one of the products according to the invention is combined with

pharmaceutically acceptable excipients, solid or liquid, suited to the composition of oral, ocular or nasal forms, or with excipients suited to the composition of the rectal forms of administration, or also with gelatinous excipients for vaginal administration. It also relates to isotonic liquid compositions containing at least one of the conjugates according to the invention, adapted to administration to mucous membranes, in particular ocular or nasal mucous membranes.

Advantageously, the vaccinating compositions according to the invention contain in addition a vehicle such as polyvinyl-pyrrolidone, which facilitates the administration of the vaccine. Instead of polyvinyl-pyrrolidone it is possible to use any other type of adjuvant in the classical sense which this expression used to connote, i.e., a substance which makes the absorption of the medicine easier or facilitates its action in the organism. As examples of other adjuvants of this latter type, mention should also be made of carboxymethylcellulose, the hydroxides and phosphates of aluminium, saponin or all other adjuvants of this type, well known to the specialist skilled in the art. Finally, if necessary, they contain an immunological adjuvant, in particular of the muramyl peptide type.

The invention also relates to pharmaceutical compositions containing as active substance at least one of the monoclonal or polyclonal antibodies previously defined in combination with a pharmaceutically acceptable vehicle.

The invention is obviously not limited to the embodiments described above as examples and the specialist skilled in the art may make modifications to them without exceeding the limits of the framework of the Claims given hereafter; in particular, some of the amino acids of the sequence of the peptides according to the invention may be replaced by

isofunctional or isosteric amino acids; for example, one or more of the following substitutions may be envisaged:

- Glu is replaced by Asp or Gln,
- Leu is replaced by Ala, etc...

It is naturally understood that the peptides which result from such substitutions consist of equivalents of the peptides more particularly claimed, provided that they themselves or oligomers or conjugates formed from these peptides exhibit similar immunogenic properties.

The invention also relates more particularly to the “chimeric proteins” which can be obtained by the procedures of genetic engineering, these chimeric proteins containing one or more of the peptide sequences of the invention, and being incorporated into or attached to a peptide fragment other than beta-galactosidase. This latter peptide fragment preferably has a molecular weight sufficient to reinforce the immunogenicity of the peptide sequences according to the invention and does not interfere immunologically with the expression of the desired immunogenicity.

Additional characteristics of the invention will also become apparent in the course of the description which follows of the conditions under which the polypeptides of the invention were obtained.

Sera derived from Europeans living in endemic areas and following a continuous prophylaxis with medicines directed against the schizonts of the blood stages (chloroquine) were selected and tested by using antigens of the sporozoite stage, the hepatic stage (LS antigen) and the blood stages. Most of these sera react with the antigens of all of the stages probably because the prophylaxis had been interrupted. Three sera taken from individuals who had resided in rural tropical Africa and who had ingested 100 ug of chloroquine per day without interruption for 23 to 26 years did not react with the antigens



of the blood stages according to the immunofluorescence assay (IFA). However, these three sera possessed high titers of antibodies directed against the sporozoites and the LSA proteins (dilution IFA 1/3200 and 1/6400, respectively).

One of the three sera just mentioned, with reduced specificity, was used to screen a genomic DNA library constructed in the bacteriophage  $\lambda$ gt 11 in the following manner:

#### 1) CONSTRUCTION OF THE GENOMIC DNA LIBRARY OF Plasmodium falciparum

The genomic DNA of clone 96 of the Thailand Tak9 strain of P. falciparum (Science, 212 137-138 (1981) was isolated by standard procedures.

Samples of 18  $\mu$ g of DNA of P. falciparum were incubated at 15°C in a 50 mM Tris HCl buffer, pH 7.5, 1 mM MnCl<sub>2</sub>, 20  $\mu$ g/ml of bovine serum albumin with variable amounts of DNAase I (Boehringer Mannheim): 5 pg for 5 minutes or 3.5 pg for 5 or 10 minutes. After addition of 5 mM EDTA (ethylenediamine tetraacetic acid), the samples of DNA are pooled and purified by extraction with a phenol / chloroform / isoamyl alcohol mixture (25 V / 24 V / 1 V), then with ether. The DNA is concentrated by precipitation with ethanol at -20°C in the presence of 2.5 M ammonium acetate.

45  $\mu$ g of DNA thus treated was methylated by means of 180 U of EcoRI methylase (Biolabs) under the conditions recommended by the supplier, with the further addition of 5 mM DTT (dithiothreitol) for 15 minutes at 37°C. After purification of the DNA as above, 10  $\mu$ g of DNA were incubated with 40 mM Tris HCl, pH 8.0, 10 mM ammonium sulfate, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.05 mM NAD (nicotinamide

adenine dinucleotide) 0.1 mM dXTP (comprising the 4 deoxynucleosides triphosphates dATP, dCTP, dGTP and dTTP) in the presence of 10 U T4 DNA polymerase (PL Biochemicals) and 10 U of *E. coli* DNA ligase (Biolabs). The DNA was purified and concentrated as above.

8 µg of DNA were then ligated with 0.4 µg of an *Eco*R1 adapter or “linker” (*Eco*R1 phosphorylated adapter marketed by Biolabs) by means of 4 U T4 DNA ligase (Biotec) in a 50 mM Tris HCl buffer, pH 8.0, 10 mM MgCl<sub>2</sub>, 20 mM DTT, 1 mM ATP, 50 µg/ml bovine serum albumin.

After incubation at 4°C for 5 hours, 2 U T4 DNA ligase are added and the reaction is allowed to proceed at 4°C for 16 hours. The tube is subjected to several cycles of freezing to -80°C / thawing to stop the reaction. The DNA is then diluted and the incubation buffer is adjusted so as to produce the conditions recommended by the supplier for the use of the enzyme *Eco*R1. 100 U of enzyme *Eco*R1 (Promega Biotec) are added and incubated for 3 hours at 37°C. The reaction is stopped by heating for 10 minutes at 60°C, and the DNA is purified and concentrated as above.

The DNA is resuspended in 100 µl of 50 mM Tris HCl buffer, pH 8.0, 1 mM EDTA and loaded on to a 5-20% sucrose gradient prepared in 25 mM sodium acetate, 10 mM EDTA and centrifuged in the Beckmann rotor SW 50.1 at 45,000 revolutions per minute for 150 minutes.

The fractions are analysed on agarose gel and those which contain the DNA fragments of a size included between about 300 bp and 2,500 bp are pooled, dialysed against 50 mM Tris HCl buffer, pH 8.0, 1 mM EDTA at 4°C. The DNA is concentrated by precipitation with ethanol. About 400ng of this DNA were ligated to 1 µg of DNA of the vector) λgt11 (Proc. Natl. Acad. Sci., USA, 80, 1194-1198 (1983) ) cut with *Eco*R1 and dephosphorylated (Proclone from Promega Biotec) in a volume of 10 µl

(in 50 mM Tris HCl buffer, pH 8.0, 10 mM MgCl<sub>2</sub> 20 mM DTT, 1 mM ATP, 50 µg/ml bovine serum albumin) by 1 U T4 DNA ligase (Biotec).

The ligation products were encapsidated in vitro in E.coli extracts prepared from the bacterial strains constructed by B. Hohn (Methods Enzymol. 68, 299) according to the procedure described by Maniatis et al. (Molecular cloning, a laboratory manual, p. 264, Cold Spring Harbor Laboratory (1982)).

About 7 millions of recombinant bacteriophages were obtained.

## 2) IMMUNOLOGICAL SCREENING OF THE BANK

The recombinant bacteriophages were spread on a culture medium containing the indicator bacteria Y 1090 at a density of 50,000 plaques per 90 mm Petri dish, and incubated at 42°C for 3 hours. A nitrocellulose filter (Schleicher & Schuell, BA 85) saturated with 0.01 M IPTG isopropyl-beta-thiogalactopyranoside (Sigma) is deposited on the dishes which are incubated at 37°C for 3 hours. When the incubations are complete, the nitrocellulose filters are removed and the Petri dishes are stored at 4°C.

The nitrocellulose filters are placed in a bath of TL buffer: 50 mM Tris HCl pH 8.0, 150 mM NaCl 5% skimmed milk, 0.05% Tween 20 (Sigma). The filters are incubated for 15 hours at 4°C in TL buffer, then twice for 15 minutes at 20°C. They are then incubated for one hour with a pool of human immune antisera directed against the antigens of all stages of development of P. falciparum, treated beforehand to deplete it of anti E.coli antibodies according to the procedure described by Ozaki et al. (J. Immunol. Methods, 89, 213-219, 1986). The pool of human antisera was used at a dilution of 1/200 in TL buffer. The incubation was carried out at 20°C for 1 hour. The filters were washed 4 times with the TL buffer, then incubated with anti-human immunoglobulin antibodies conjugated to horseradish

peroxidase (Biosys) and iodinated with  $^{125}\text{I}$  for 1 hour at 20° C. After several washings with TL buffer, followed by 50 mM Tris HCl buffer, pH 8.0, 150 mM NaCl, the enzymatic activity of the peroxidase is revealed (Ozaki et al., previously cited), the filters are dried in air and autoradiographed using Kodak film Royal X-OMat AR with an amplifying screen.

A collection of about 1200 clones of recombinant bacteriophages was established by selecting the lysis plaques corresponding to the positive signals. These clones were then subjected to a second cycle of immunological screening by using this time one of the three human sera previously described and exhibiting few or no antibodies directed against the erythrocytic forms of P. falciparum and a high titer against the sporozoite and hepatic forms of the parasite. This immunological screening was carried out according to the protocol described above. This serum has led to the identification of about recombinant 120 clones out of 1200 tested.

The human antibodies which react with the antigenic determinants expressed by the recombinant clones were purified by means of their affinity for the recombinant proteins according to the procedure described by Ozaki et al. (previously cited). These specific antibodies were incubated with preparations of parasites at different stages of development (sporozoite, hepatic stage or erythrocytic stages), and the reaction was studied by means of indirect immunofluorescence.

The recombinant clones on which the antibodies specific for the hepatic stage are retained by affinity and which thus express determinants intrinsic to this stage were studied: they are the clones DG 307, DG 199 and DG 145. These specific antibodies of these three clones react specifically with the hepatic schizonts such as can be obtained after infection of human or monkey hepatocytes by sporozoites of P. falciparum; the localization of

the fluorescence was determined to be identical with that considered to be characteristic of LSA.

The species- and stage-specificity of the 3 clones DG 145, DG 199 and DG 307 were tested in the following manner. Firstly, it was determined that the same antibodies purified by affinity and which react by IFA (or also which are IFA-positive) with LSA do not react with dried or moist preparations of sporozoites, nor with the antigens of the blood stages, whether they are tested by IFA with parasites fixed in acetone or by immunoblotting by using proteins of all of the stages extracted with SDS. The antibodies purified by affinity do not react with the antigens of the hepatic stage of P. yoelii nor with the hepatic schizonts of P. vivax prepared from Saimiri sciureus monkeys.

Secondly, the recombinant proteins of DG 145, DG 199 and DG 307 do not react with the sera obtained from two patients suffering from malaria (caused by P. falciparum) by accidental transfusion and which, by definition, thus do not have antibodies against the antigens specific for the earlier stages (sporozoites and antigens of the hepatic stage). These proteins do not react with two monoclonal antibodies which recognize the CS tetrapeptide, with the sera of mice immunised with the recombinant CS antigens R32t and 32 (Science, 228 958 (1985)). Furthermore, the recombinant proteins did not react with human antisera directed against P. vivax (although the sera were positive with the hepatic schizonts of P. vivax, P. ovale and P. cynomolgi (Ann. Soc. Belg. Med. Trop., 60, 348 (1980)) when they are tested by the technique of immunodot blots, whereas they are positive with all of the human anti-P. falciparum sera tested.

Within this sub-population of 120 clones mentioned above, an immunological screening procedure using antibodies purified by affinity to

the clone DG307 (or 145, or 199) leads to the detection of about 40 clones out of 120 apparently exhibiting the characteristic epitope of the LSA and defined by the basic structure of 17 amino acids cited above.

Similarly, identification procedures based on hybridization with the aid of DNA fragments from the same clones (DG307) make it possible to identify these repetitive structures in the same clones as those identified by the immunological assays.

A complementary screening of the sub-population of 40 clones belonging to this family of the LSA antigen was used to identify other parts of the gene containing sequences distinct from those defined by the repeats of 17 amino acids. For this complementary screening sera identified as not reacting with the repeats of the 17 amino acids but positive in indirect immunofluorescence with the peripheral structure of the hepatic schizont in which the LSA antigen is situated, were employed. Within the family of the 40 clones of the LSA, these sera were found to be positive for several of them, and one of the clones containing the largest insert, designated DG536, was selected and studied in detail.

Other clones, DG538, DG750 and DG443 were studied. The clones DG750 and DG443 contain the major part of the non repetitive 5' sequence of the LSA gene.

The insert of 951 base pairs was purified and recloned in the bacteriophage M13 mp19. The DNA sequence and the genomic organization of the LSA gene were then determined. Figure I shows that the clone contains a sequence of 209 amino acids at the 5' end corresponding to a series of 12 repeats of 17 amino acids, similar to that described in the article by Guérin-Marchand et al. (Nature, mentioned above) and then contains a set of 106 amino acids, the structure of which is not repetitive.

As can be seen in Figure 1, the motif of 7 amino acids is in two repeats (cf. motif corresponding to the positions 35 to 51, and that corresponding to the positions 137 to 153 of figure 1) identical with that described in the article by Guérin-Marchand et al. and the other repeats exhibit a substitution of a leucine by an arginine (cf. positions 8, 59, 76, 110, 127, 161, 178 and 195 of Figure 1), a substitution of a glutamic acid by an aspartic acid (cf. positions 23 and 91 of Figure 1) as well as a substitution of a serine by an arginine (cf. position 205 of Figure 1).

It was possible to measure the size of the native protein of the LSA in the parasite after surmounting great difficulties. Hepatic schizonts of P. falciparum were produced in vitro by infection of human hepatocytes from a primary culture with salivary glands of mosquitoes containing sporozoites according to the procedure described in Mazier et al. (Science No. 227, p440, 1985). After 7 days of culture, the infected cells are recovered and used to prepare an extract analysed on polyacrylamide gel containing SDS and transferred to nitrocellulose. The hepatic antigens are then revealed by an antibody purified by affinity to the repeats of the 17 amino acids of the LSA already mentioned. These antibodies label a protein of molecular weight of 200,000 Daltons.

The hybridization of the clone DG307 with the DNA fragments of P. falciparum obtained by digestion with a restriction enzyme, the Mung bean nuclease, which under the conditions described by McCutchans (NAR, 16, 14, 6883-6896, 1988) is capable of cutting each end of the genes of the parasite, reveals a unique band of molecular weight of 5 K bases consistent with the size of the native protein measured in the parasite extract.

In electron microscopy of the hepatic schizonts obtained by injection of sporozoites of chimpanzees, the labelling of the anti-LSA antibodies

purified by affinity, visualized by a second antibody labelled with colloidal gold, reveals that the LSA molecule is distributed: a) at the stage of the hepatic trophozoite and the young schizont, in vacuoles containing granules which open and discharge their contents into the parasitophorous vacuole, b) at the stage of the 5 days old immature schizont, at the periphery of the hepatic schizont in the granules present in the parasitophorous vacuole of the parasite, c) in the 6 to 7 days old mature schizonts, in the parasitophorous vacuole as well as between the pseudo cytomers of the schizont, then finally surrounding the merozoites in process of formation.

The study of the immunological response of subjects exposed to malaria as well as animals immunized with the recombinant and/or synthetic proteins makes it possible to specify the biological function of the LSA protein and of certain segments of this protein, in particular those contained in the synthetic peptides.

The immunization of mice with the LSA-R-NR proteins and the study of the response of the lymphocytes of these mice, as well the immunization with the LSA-R peptides of mice of different haplotypes with peptides LSA-R, LSA-J and LSA-NR, and finally the study of the responses of the lymphocytes of the subjects exposed to malaria towards the LSA-R peptides had shown that a T epitope for man and the mouse is not defined by the repetitive part of the LSA molecule. More detailed studies show the existence of a T epitope in the LSA-R peptide. As had been shown previously, the LSA-R peptide constitutes an excellent B epitope for man and the mouse, defined by the repetitive part of the LSA molecule, and the complementary results obtained since in more than 500 individuals exposed to malaria show that this epitope is recognized by the antibodies of about



95% of the subjects studied, in Senegal, Upper Volta, Madagascar and Kenya.

The preliminary study of the lymphocytes of 5 adult African subjects exposed to malaria, subsequently confirmed by the detailed study of the response of the peripheral lymphocytes of more than 200 adult African subjects exposed to malaria as well as the lymphocytes of chimpanzees (*Pan troglodytes*) immunised by the recombinant protein LSA-R-NR (clone DG536), revealed that a T epitope of the LSA molecule is defined by the amino acid sequence contained in the synthetic peptide LSA-NR. Two other T epitopes are contained in the sequences of the synthetic peptides LSA-J and LSA-R (in total 39% of positive responses to the T epitopes of the LSA in Madagascar and 83% in Senegal). Proliferative responses of the human lymphocytes and the lymphocytes of an immunised chimpanzee were observed after stimulation by these three peptides, a) in the chimpanzee 60% of the lymphocytic lines obtained are of the CD8<sup>+</sup> phenotype, b) in the mouse, the injection of one or other of these peptides makes it possible immunologically to “prime” the immune system of the mouse and to obtain the production of a high level of antibodies against the B epitope of the LSA-R peptide after the injection of the recombinant protein LSA-R-NR. The identification of those epitopes capable of stimulating the T lymphocytes is of great importance in as much as it has been established in the malaria of rodents that the protection induced by irradiated sporozoites is dependent on the production of lymphocytes cytotoxic for the infected hepatocyte, and capable of destroying them.

Furthermore, the study of 120 sera of African subjects also shows that the peptide LSA-NR defines a B epitope, distinct from that which is found in the repeats, recognized by about 65% of the subjects studied.

On the other hand, the peptide LSA-TER does not constitute a significant B epitope, it is rarely recognized by the antibodies of the subjects studied.

The potential usefulness of the T epitopes of the LSA, in particular those contained in the non-repetitive part, is, in addition strongly reinforced by the results obtained in the chimpanzee:

in the chimpanzee which has been immunised by three injections at intervals of 15 days of a mixture of two recombinant proteins adsorbed on alum, the recombinant protein LSA-R-RN (clone DG536) on the one hand and the recombinant protein designated DG729S (clone DG729S which forms part of the 120 clones mentioned above) on the other, it was possible to obtain several significant results:

- the production of antibodies specific for each of the two recombinant molecules, i.e. which react with the protein LSA-R-NR and the synthetic peptides, as well as with the recombinant protein 729S, was detected.
- a specific proliferative response of the lymphocytes was obtained towards the peptide LSA-NR and also to the peptides LSA-J and LSA-TER. Sixty percent of the proliferating lymphocytes are of the CD8<sup>+</sup> phenotype, which corresponds in particular to cytotoxic T lymphocytes.
- After immunization of the chimpanzees, a test infection by intravenous injection of 28 millions of sporozoites of P. falciparum was carried out on an immunized chimpanzee and in a control chimpanzee (which received an unrelated control recombinant protein) and liver biopsies were made on day 6 after injection. The examination of the biopsies showed the existence of a cellular reaction, lympho-monocytic, around the hepatic schizonts, infiltrating the schizonts and capable of destroying them. Such images were not observed in the chimpanzee which had received the control antigen.

This cellular reaction reveals the existence of T epitopes in the injected molecules 729S and LSA-R-NR (DG536) which are capable of being expressed in the injected molecules, capable of being expressed by the hepatic schizonts and of inducing a cellular afflux; this result is in agreement with the results of the lymphocytic proliferation assays; finally it shows that the immune response induced by the injected recombinant proteins is capable, upon penetration of the parasite, of inducing a cellular afflux, itself capable of contributing to the defence of the organism by destroying the parasites located within the hepatocytes.

A specific proliferative response of the lymphocytes was also obtained towards the peptides 729S-NRI and 729S-NRII.

The cytolytic capacity of the lymphocytes of this immunized and protected chimpanzee was measured in vitro in the following manner:

lymphocyte lines were produced by in vitro stimulation with the peptides LSA-NR and LSA-J as well as with 729-NRI and NRII in the presence of interleukin 2. These lines were maintained for 4 weeks by restimulation in the presence of autologous mononucleated cells from the same chimpanzee by the same peptides and interleukin 2. A cytotoxicity test was carried out by incubating the irradiated peripheral mononucleated cells of the chimpanzee with the same peptides, then after labelling with chromium 51, by incubating these cells with the lines produced. Release of the chromium 51 reflecting the destruction of the target cells was observed. These results demonstrate that the T epitopes, defined above, are capable of activating the cytotoxic T lymphocytes specific for the polypeptide sequences in question.

The antigenic and/or immunological specificities of the polypeptides of the invention are the following:

- the polypeptide 536:

\* is recognized:

° by antibodies originating from subjects with malaria,

° by sera of chimpanzees immunized with the recombinant complete protein 536,

\* co-reacts with the polypeptides described in Guérin-Marchand et al. (Nature, cited above) by the intermediary of the repeat sequences,

\* induces the function of antibodies (presence of B epitopes)

\* induces proliferative responses of lymphocytes (presence of T epitope) in the chimpanzee and in man.

- the polypeptides NR and TER include a major T epitope and a B epitope for man as for the immunized animal (mouse and chimpanzee),

- the polypeptide LSA-J includes a B epitope distinct from that of the 17 amino acid repeat probably on account of the substitution of S by R, and contains a T epitope recognized in man and the chimpanzee.

The recombinant protein 729S:

- is recognized

\* by antibodies of subjects exposed to malaria,

\* by sera of chimpanzees and mice immunized by the protein 729S

- is better recognized by the individuals capable of resisting infection by malaria than by those who do not resist (in the north of Senegal, the inventors have administered a radical course of treatment of chloroquine to 100 individuals, and followed the repositivation of the blood during the period of transmission, from September to December a) in the subjects who are not positive, no antibody against the protein 729S was detected, b) in more than half of those who had not become positive again, there existed a high titer response to the protein 729S).

- is recognized by the sera of three individuals who had been vaccinated by multiple injections of sporozoites of P. falciparum and who had resisted a test injection of non-irradiated virulent sporozoites, but is not recognized by the sera of four other individuals who had been vaccinated by multiple injections of sporozoites irradiated with high doses of radiation and who had not resisted the same test injections of non-irradiated sporozoites. This recognition relates in particular to the reaction of the antibodies with the polypeptide 729S-NRII. Hence there exists a tight correlation between the immune response directed against the molecule 729S and the protection induced against malaria in man.

The polypeptides 729S-NRI and 729S-NRII bear additionally major T epitopes which are recognized by the majority of these subjects whose lymphocytes were studied recently in Madagascar and Senegal (18 out of 20 positives studied in Madagascar and 26 out of 46 studied in Senegal).

The polypeptide 729S-R also contains a T epitope recognized by a high proportion of individuals of Senegalese and Madagascan origin (30 to 60% of the individuals studied) but in addition it defines a major B epitope of the molecule because the antibodies of 96 to 100% of the subjects studied in Senegal, Cameroon and Madagascar recognize this peptide and because the antibody titer observed is extremely high.

It will immediately be apparent to the specialist skilled in the art that in the nucleotide sequences mentioned above, some of the nucleotides may be replaced by others on account of the degeneracy of the genetic code without the peptides being modified in any way. All of these nucleotide sequences as well as those which code for polypeptides which differ from former by one or more amino acids without their intrinsic immunogenic activity being similarly modified, form part of the invention. Naturally, the

same holds for the nucleotide sequences which may be reconstituted and which are capable of coding for oligomers such as defined hereafter. The monomeric motifs are linked directly end to end or through the intermediary of peptide sequences without any effect on the immunogenic properties of the oligomers thus formed.

Bacteria harbouring the above-mentioned clones DG199 and DG307 were deposited with the Collection Nationale des Cultures de Microorganismes at the Pasteur Institute in Paris (CNCM) on 22 July 1986 under the numbers I-580 and I-581, respectively. Bacteria harbouring the clone DG145 was deposited on 15 September 1986 under the number I-606. The clones DG 536 and DG 729 were deposited on 17 January 1991 under the number I-1027 and on 17 January 1991 under the number I-1028, respectively.

In the preceding formula, use is made of the international nomenclature which designates each of the naturally occurring amino acids by a single letter, in particular according to the table of correspondances which follows:

M	Methionine
L	Leucine
I	Isoleucine
V	Valine
F	Phenylalanine
S	Serine
P	Proline
T	Threonine
A	Alanine
Y	Tyrosine

H	Histidine
Q	Glutamine
N	Asparagine
K	Lysine
D	Aspartic acid
E	Glutamic acid
C	Cysteine
W	Tryptophan
R	Arginine
G	Glycine